Transforming Growth Factor-β1 Overproduction in Prostate Cancer: Effects on Growth *in Vivo* and *in Vitro*

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We found previously that transforming growth factor-β1 (TGFβ1) mRNA levels are markedly elevated in rat prostate cancer (Dunning R3327 sublines) compared to levels in normal prostate. Our goal was to determine whether elevated expression of TGFβ1 is biologically relevant to prostate cancer growth *in vivo*. We chose as our model the R3327-MATLyLu prostate cancer epithelial cell line, which produces metastatic anaplastic tumors when reinoculated *in vivo*. Our approach was to stably transfect MATLyLu cells with an expression vector that codes for latent TGFβ1 and to isolate subclones of cells that overexpressed TGFβ1 mRNA. We also isolated a subclone of MATLyLu cells transfected with a control vector lacking the TGFβ1 cDNA insert. We then studied the growth of these cells *in vivo* and *in vitro*. Twenty days after sc inoculation of 10^6 cells *in vivo*, TGFβ1-overproducing MATLyLu tumors were 50% larger, markedly less necrotic, and produced more extensive metastatic disease (lung metastases in 73% of all lobes and lymph node metastases in 88% of animals) compared to control MATLyLu tumors (lung metastases, 21%; lymph node metastases, 7%). Thus, TGFβ1 produced *in vivo* is biologically active and can promote prostate cancer growth, viability, and aggressiveness, perhaps via effects on the host and/or on the tumor cells themselves. When followed *in vitro*, TGFβ1-overproducing cells became growth inhibited, but this effect was transient as cells subsequently resumed proliferating. Growth inhibition was due to TGFβ, because it could be prevented by TGFβ-neutralizing antibody. Therefore, prostate cancer cells can activate and respond to secreted latent TGFβ1, and although the cells are transiently inhibited *in vitro*, there is no net inhibition of growth. The ability of the cells to respond to endogenously produced TGFβ1 suggests that TGFβ1 overexpression enhances tumor growth *in vivo* at least in part via an effect of TGFβ1 on the tumor cells themselves. (Molecular Endocrinology 6: 15–25, 1992)

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INTRODUCTION

Transforming growth factor-β1 (TGFβ1), a 25-kilodalton (kDa) dimeric polypeptide, is widely referred to as a prototypical epithelial cell inhibitor (1, 2). Yet, TGFβ1 expression in many cancers is higher than that in their normal tissue counterparts (3–9). For example, we recently reported that TGFβ1 mRNA levels were dramatically elevated in rat prostate adenocarcinomas (Dunning R3327 sublines grown *in vivo*) compared to those in normal prostate and were higher the greater the degree of anaplasia and the faster the growth rate (3). This presents an apparent paradox if one views TGFβ1 strictly as an epithelial inhibitor. However, TGFβ1 also has potential tumor growth-promoting properties; for example, TGFβ1 can stimulate angiogenesis (1, 2, 10), suppress the immune system (11, 12), and stimulate invasion and metastatic potential (13).

Speculation aside, elevated mRNA expression does not necessarily reflect the production of elevated levels of biologically active, secreted TGFβ1 protein. TGFβ1 is secreted by cells in an inactive latent form that does not bind to TGFβ receptors; latent TGFβ1 must be activated to be biologically active (1, 2, 14, 15). Thus, although prostate tumors produce large amounts of TGFβ1, this would be of no consequence if it remained in the latent form. There are indeed many tumor cell types that cannot activate latent TGFβ1, that lack TGFβ receptors, or that are insensitive to TGFβ1 (1, 14).

Our goal, therefore, was to determine whether elevated expression of TGFβ1 was biologically relevant to prostate cancer growth *in vivo*. The approach we chose was to stably transfect TGFβ1 cDNA (which codes for latent TGFβ1) into prostate cancer cells and to isolate a subclone of cells that overexpress TGFβ1 mRNA. These TGFβ1-overproducing cells could be studied *in vitro* and also could be reinoculated *in vivo* to produce

sc tumors. The model we chose was the Dunning R3327-MATLyLu rat prostate adenocarcinoma subline, which is serially transplantable, anaplastic, and metastatic (16). An *in vitro* cell line of this tumor has been established, and cells passaged *in vitro* can be reinoculated *in vivo* to produce a tumor with properties like those of the original *in vivo* tumor (17).

The control nontransfected MATLyLu tumor already produces high levels of TGFβ1 mRNA (3). We reasoned that if this TGFβ1 had no effect on tumor growth, then engineered overexpression of even higher levels of TGFβ1 would be of no consequence, and tumor behavior of TGFβ1-overproducing transfected cells would be no different from that of control cells. On the other hand, if TGFβ1 produced by prostate cancer cells affected tumor growth, then overexpression might enhance this effect. Thus, if TGFβ1 were acting as a typical epithelial cell inhibitor, then TGFβ1 overexpression should inhibit or slow tumor growth (compared to control tumors). In contrast, if TGFβ1 was a positive modulator of prostate cancer, then TGFβ1 overexpression should enhance tumor growth.

Our approach differs from that of other studies of the effects of TGFβ1 on tumor epithelial cells. The effects of TGFβ1 on prostate cancer epithelial cells have been reported previously (18–22), but those studies were carried out only *in vitro* and used activated TGFβ1 that was added exogenously. To date, therefore, the role of endogenously produced TGFβ1 in prostate cancer growth *in vivo* has not been documented. A unique aspect of our approach is that we are able to investigate the role of endogenously produced TGFβ1 and study its effects on cells *in vivo* and *in vitro*.

We found that TGFβ1-overproducing MATLyLu tumor cells produced significantly larger and less necrotic tumors *in vivo* and produced more extensive metastatic disease than did control MATLyLu cells. These dramatic effects indicate that endogenously produced TGFβ1 is biologically active *in vivo* and can promote prostate cancer growth, viability, and aggressiveness. These data are the first to illustrate a net growth-promoting effect of TGFβ1 on an epithelial tumor *in vivo*. Proliferative behavior *in vitro* indicated that the tumor cells themselves could activate secreted latent TGFβ1 and respond, although the response was transient inhibition. These data illustrate the value of *in vivo* studies in demonstrating that TGFβ1 is not acting as a prototypical epithelial cell growth inhibitor in prostate cancer.

RESULTS

TGFβ1 Overexpression in MATLyLu Cells Transfected with TGFβ1 cDNA

MATLyLu prostate cancer epithelial cells were cotransfected with the expression vectors pSVTGFβ1 (which codes for latent TGFβ1) and pZipneo (which confers G418 drug resistance). Long term growth of transfected cells in medium containing G418 allowed selection of drug-resistant colonies that had stably integrated the pZipneo gene into the cellular genome. Stable integration also of the pSVTGFβ1 vector was confirmed by probing a Southern DNA blot for the presence of specific plasmid sequences (data not shown). Stable integration of the murine TGFβ1 cDNA, however, did not necessarily mean that the subclone was producing high levels of TGFβ1. To identify subclones that produced high levels of TGFβ1 mRNA, poly(A)$^+$ RNA was isolated from subclones that contained the murine TGFβ1 insert and adjacent vector sequences, and TGFβ1 mRNA levels were assayed by Northern blot analysis. Figure 1 (*right lane*) shows one subclone of cells transfected with the TGFβ1 expression vector (referred to as pSVTGFβ1, subclone 2B5) that produced markedly higher levels of TGFβ1 mRNA than nontransfected MATLyLu cells (Fig. 1, *left lane*). Both cell lines, in contrast, expressed similar amounts of actin mRNA (Fig. 1). The transfection procedure itself did not affect TGFβ1 expression, since MATLyLu cells transfected with the control vector pSG5, which lacks a TGFβ1 insert (referred to as MATLyLu-pSG5 cells) produced TGFβ1 mRNA levels similar to those produced by nontransfected cells (Fig. 1, compare *left and middle lanes*). mRNA transcripts of the transfected murine TGFβ1

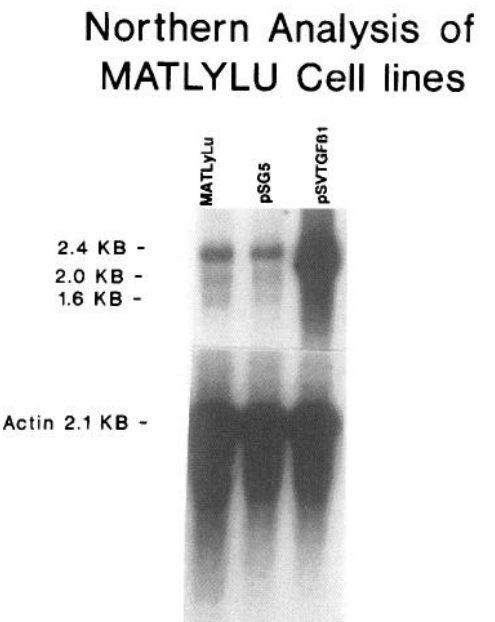

Fig. 1. Northern Analysis of MATLyLu Cell Lines

Poly(A)$^+$ mRNA was isolated from cell monolayers, electrophoresed in 1.7% agarose, transferred to nylon, and hybridized with a [^{32}P]dCTP-labeled 1.6-kb murine TGFβ1 cDNA probe. TGFβ1 mRNA was markedly more abundant in MATLyLu-pSVTGFβ1 cells (*right lane*) than in nontransfected MATLyLu cells (*left lane*) or MATLyLu-pSG5 cells transfected with a control vector lacking TGFβ1 cDNA (*middle lane*). The same blot was stripped and rehybridized with an actin probe (*bottom panel*), indicating that equal amounts of mRNA were loaded per lane (5 μg).

gene were 2.3 kilobases (kb) and could be distinguished from the predominant 2.4-kb transcript of the endogenous rat TGFβ1 gene. However, because TGFβ1 is virtually 100% conserved between mice and humans (23), it is likely that the effects of the product of transfected murine TGFβ1 cDNA would be identical to those of the endogenous rat TGFβ1 expressed by MATLyLu cells.

TGFβ1 Overexpression Promotes Tumor Growth *in Vivo*

We, thus, now had MATLyLu cell lines that differed in their level of TGFβ1 mRNA expression. To determine whether this difference affected tumor cell behavior, we investigated tumor growth *in vivo*.

Nontransfected MATLyLu cells, control transfectants (MATLyLu-pSG5), and TGFβ1-overproducing cells (MATLyLu-pSVTGFβ1) were injected into the flanks of adult male rats, and tumor size was measured at frequent intervals (Fig. 2). For the first 8 days of growth *in vivo*, tumor volumes were not significantly different among the three groups. However, by 15 days, TGFβ1-overproducing MATLyLu-pSVTGFβ1 tumors were significantly larger (18.6 ± 1.6 cc; n = 18) than control MATLyLu tumors (13.0 ± 1.0 cc; n = 16) or MATLyLu-pSG5 tumors (11.5 ± 1.1 cc; n = 17; $P < 0.005$). Also at 17 and 20 days, TGFβ1-overproducing tumors were 50% larger than either MATLyLu or MATLyLu-pSG5 tumors ($P < 0.005$).

Animals were killed on day 20. Control tumors (Fig. 3A) had markedly more central necrosis than TGFβ1-overproducing tumors (Fig. 3B). Only a thin external rim of viable tumor was evident in control MATLyLu tumors (Fig. 3A), whereas the bulk of each MATLyLu-pSVTGFβ1 tumor was viable (Fig. 3B). Therefore, although the volume of MATLyLu-pSVTGFβ1 tumors was 1.5 times that of control tumors, the amount of viable tissue was probably much more than 1.5 times greater. Northern RNA analysis confirmed that the MATLyLu-pSVTGFβ1 tumors were expressing much higher levels of TGFβ1 mRNA than were control MATLyLu tumors (Fig. 4).

These data provide compelling evidence that TGFβ1 overexpression indeed affects MATLyLu tumor behavior. These are the first data that illustrate a net growth-promoting effect of TGFβ1 on an epithelial tumor *in vivo*.

TGFβ1-Overproducing Tumors Have a Higher Metastatic Rate

When rats were killed on day 20, control rats with MATLyLu or MATLyLu-pSG5 tumors had visible lung

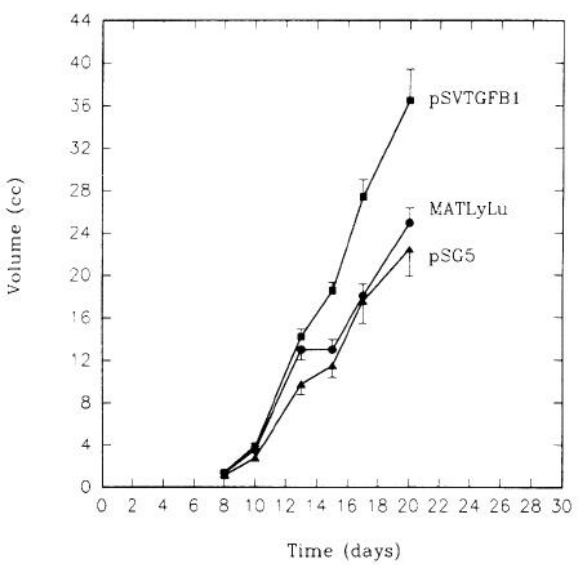

Fig. 2. *In Vivo* Growth of MATLyLu Tumor Sublines

Cell monolayers were washed in Hanks' buffer, trypsinized, and resuspended in Hanks' buffer. Tumor cells (1×10^6) were inoculated into the flanks of male Copenhagen rats on day 0. Tumors formed by MATLyLu (●), MATLyLu-pSG5 (▲), and MATLyLu-pSVTGFβ1 (■) cells were measured in two dimensions by calipers. Tumor volumes and doubling times were calculated as previously described (3). Each *point* represents the mean ± SEM volume of 16–20 tumors.

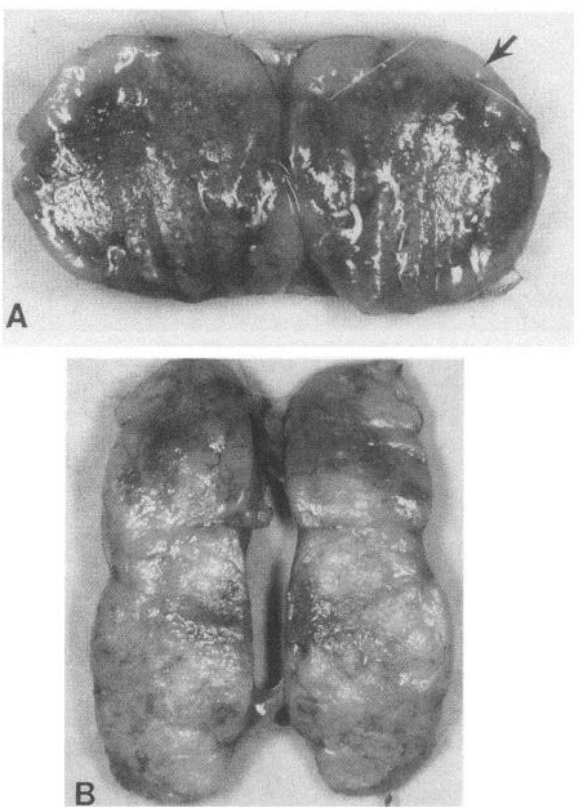

Fig. 3. Macroscopic Cross-Section of MATLyLu (A) and TGFβ1-Overproducing MATLyLu-pSVTGFβ1 (B) Tumors

Twenty days postinoculation, animals were killed, and tumors were bisected and inspected for central necrosis. This figure compares a 12.6-cc MATLyLu tumor (A) and a 23.8-cc MATLyLu-pSVTGFβ1 tumor (B). Control MATLyLu tumors had markedly more central necrosis, and only a peripheral rim of viable tissue (*arrow*). In contrast, TGFβ1-overproducing MATLyLu-pSVTGFβ1 tumors had very little necrosis, and the bulk of the tumor appeared viable.

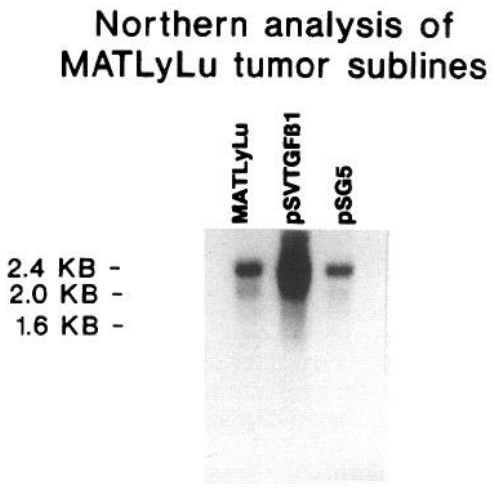

Fig. 4. Northern Analysis of MATLyLu Tumor Sublines

Poly(A)$^+$ mRNA was isolated from the MATLyLu tumor sublines, electrophoresed, transferred to nylon, and hybridized with TGFβ1 cDNA. TGFβ1 mRNA transcripts were much more abundant in MATLyLu-pSVTGFβ1 tumors (*middle lane*) than in control nontransfected MATLyLu tumors (*left lane*) or MATLyLu-pSG5 tumors (*right lane*). This confirms that the MATLyLu-pSVTGFβ1 tumors growing *in vivo* were still overproducing high levels of TGFβ1.

Table 1. Quantitation of Metastatic Disease in Tumor-Bearing Animals

Tumor Subline[a]	Primary Tumor Vol (cc)	Lung Metastases (% of lobes)[b]	Lymph Node Metastases (% of animals)[c]
MATLyLu	25.0 ± 1.4	26 ± 14	14
MATLyLu-pSG5	22.4 ± 2.5	17 ± 3	0
MATLyLu-pSVTGFβ1	36.5 ± 2.9	73 ± 12	88

[a] Cells (10^6) of each subline were injected sc in the flank. Twenty days later, animals were killed, primary tumor volume was measured, and visible metastases were quantitated, as described in *Materials and Methods*.

[b] The percentage of all five lung lobes that contained one or more visible metastases was determined for each rat; the mean percentage (±SEM) for the number of animals in each group is shown.

[c] Gross lymph node metastases were found in one of seven animals with MATLyLu tumors (nontransfected), none of seven with MATLyLu-pSG5 tumors (control transfectants), and seven of eight animals with TGFβ1-overproducing tumors (pSVTGFβ1 transfectants).

metastases in 26% or 17% of all lung lobes, respectively. In striking contrast, MATLyLu-pSVTGFβ1 tumor-bearing animals had lung metastases in 73% of all lung lobes. Lymph node metastases were found in 14% or 0% of animals with control tumors, whereas 88% of animals with TGFβ1-overproducing tumors had lymph node metastases (Table 1).

According to previous studies, virtually all animals with control MATLyLu tumors eventually develop lymph node and lung metastases (16, 17). It is apparent, therefore, that we terminated our experiment before metastases were visibly apparent in all animals. However, by doing so, we were able to detect a dramatically higher rate of metastasis in TGFβ1-overproducing tumor-bearing animals. If we had waited until all control tumors had metastasized, we probably would have missed seeing this effect of TGFβ1. These data further support a role of TGFβ1 overexpression in enhancing MATLyLu tumor growth.

A second subclone of TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells, subclone 1C6, was isolated which produced TGFβ1 mRNA levels similar to those in subclone 2B5 (see Fig. 1, *right lane*). Like subclone 2B5, subclone 1C6 also produced tumors that were significantly larger and more aggressive (produced metastases sooner) than control MATLyLu transfected with the pSG5 vector that lacks TGFβ1 cDNA. The similar behavior of two independent subclones of TGFβ1-overproducing MATLyLu-pSVTGFβ1 transfectants confirms that enhanced tumor growth and aggressiveness were, in fact, due to TGFβ1 overexpression, rather than to the transfection procedure itself.

Prostate Cancer Cells Can Activate and Respond to Secreted Latent TGFβ1

The mechanism by which TGFβ1 overexpression enhanced MATLyLu tumor growth *in vivo* may have involved effects of TGFβ1 on the tumor cells themselves and/or effects of TGFβ1 on the host. To investigate whether endogenously produced TGFβ1 had direct effects on prostate cancer cells, we studied the *in vitro* proliferative behavior of control and TGFβ1-overproducing cells in monolayer culture.

TGFβ1 is secreted in a latent form and must be activated in order to elicit a cellular response. We wished to address two questions. 1) Do prostate cancer cells activate latent TGFβ1? 2) Is their growth rate affected by TGFβ1? Because we wished to study the effect of TGFβ1 produced by the cells themselves, our experimental strategy was to grow the cells in serum-free medium and not to change the medium during the course of the experiment. We used serum-free medium to avoid the confounding effects of other serum growth factors on cell proliferation and to avoid the binding of secreted TGFβ1 to serum proteins. The medium was not changed, so that TGFβ1 secreted by the cells could accumulate to a concentration high enough to affect the cells, if they were capable of responding. If the cells in culture were not able to activate latent TGFβ1, we wished to determine whether they could, nevertheless, respond to activated TGFβ1. Therefore, to activate latent TGFβ1 secreted by the cells, conditioned medium was removed, heated to 90 C for 5 min (24), and, after cooling to 37 C, reintroduced to the cultures. Thus, cell proliferation in heat-activated medium was compared to that in unheated medium.

Nontransfected MATLyLu cells and control transfected MATLyLu-pSG5 cells had identical growth properties (Fig. 5). For both cell lines, cell proliferation was

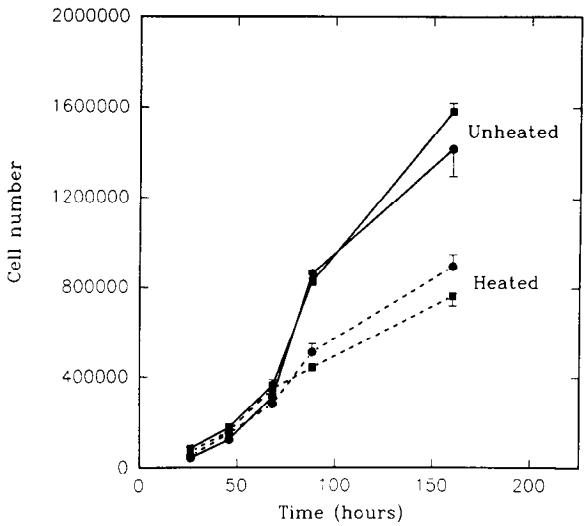

Fig. 5. *In Vitro* Growth of Nontransfected and Control Transfected Cell Lines

Nontransfected MATLyLu cells (●) and control transfected MATLyLu-pSG5 cells (■; *i.e.* cells transfected with control vector lacking TGFβ1 cDNA) were grown in unheated medium (*solid lines*) or in heat-activated medium (*dotted lines*). Cells were grown as monolayers on plastic in serum-free medium, and the medium was not changed over the course of the experiment. Each *point* represents the mean ± SEM number of cells in triplicate T-25 cell culture flasks. Heat-activated medium was prepared by heating conditioned medium at 90 C for 5 min to activate all secreted latent TGFβ1. Medium was then cooled to 37 C and reintroduced into the original T-25 flasks. Heating was repeated at each time point.

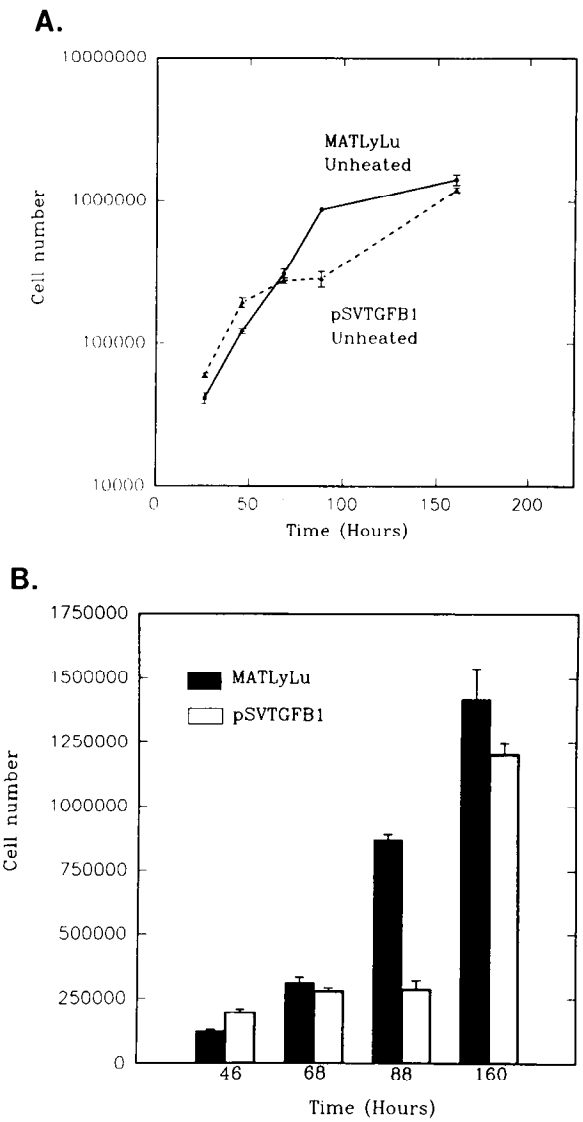

Fig. 6. *In Vitro* Growth of TGFβ1-Overproducing MATLyLu-pSVTGFβ1 Cells in Unheated Medium

Transfected TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells were grown in unheated medium (*dashed line*). Nontransfected MATLyLu cells (*solid line*) are shown for comparison. The experimental design was exactly as described in Fig. 5. In A, cell number is plotted on a log scale to illustrate exponential growth rates. In B, cell number at selected time points is plotted on a linear scale to aid comparison between the cell lines.

slower in heat-activated medium than in unheated medium. By 88 h, cell number in heat-activated conditioned medium was only 50% of that in unheated medium (Fig. 5). Thus, MATLyLu and MATLyLu-pSG5 cells became growth inhibited by a heat-activated factor secreted into the medium. That growth was not affected by heat-activated medium at early time points suggests that the concentration of secreted factor had not yet reached an inhibitory level. Although this secreted factor presumably was TGFβ1, we did not rule out the possibility that growth inhibition may have been due to heat inactivation of growth stimulatory secreted factors.

In unheated medium, cell proliferation was exponential for the first 88 h, but then slowed [cell number plotted on arithmetic scale in Fig. 5 (*solid lines*) and on log scale in Fig. 6A (*solid line*)]. Because cell proliferation was also slowed by growth in heat-activated medium (Fig. 5), it is tempting to speculate that the slowing of proliferation in unheated medium may have been due to cell-mediated activation of this secreted factor (latent TGFβ1), rather than to nutrient depletion.

TGFβ1 overexpression had dramatic effects on growth *in vitro* (Fig. 6). In unheated medium, the proliferation rates of TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells and nontransfected MATLyLu cells were similar until 46 h, after which the proliferation rate of MATLyLu-pSVTGFβ1 cells slowed dramatically (Fig. 6A). Between 46 and 88 h, the number of TGFβ1-overproducing cells increased only 1.47-fold, while the number of nontransfected MATLyLu cells increased 7-fold (Fig. 6B). Because TGFβ1-overexpressing cells became inhibited relative to nontransfected cells, we inferred that this effect was due to TGFβ1, and because this effect occurred in unheated medium, we inferred that the transfected cells themselves were able to activate latent TGFβ1. The inhibition was transient, however, as MATLyLu-pSVTGFβ1 cells resumed proliferating after 88 h; by 160 h, the total cell number was

similar to that of nontransfected MATLyLu cells (Fig. 6).

These data illustrate important features of our experimental design. Only by counting cell number at multiple time points were we able to detect this complex growth pattern of TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells. Had we measured cell number only on day 1, 2, or 7 we would have concluded that TGFβ1 overexpression had no effect on MATLyLu prostate cancer cell growth *in vitro*. If we had measured cell number on day 3 or 4 we would have observed growth inhibition, but we would have missed the transient nature of this effect. In addition, by allowing secreted factors to accumulate in the medium, we may infer that a threshold concentration must be reached before cell proliferation is affected. Had we changed the medium at frequent intervals, the concentration of this secreted inhibitor would not have reached this critical level.

When the conditioned medium of TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells was heated to activate the entire secreted pool of latent TGFβ1, proliferation was immediately and completely inhibited (Fig. 7), presumably the result of a high concentration of secreted TGFβ1. However, this growth inhibition was transient; after 46 h, MATLyLu-pSVTGFβ1 cells grown in heat-activated medium resumed proliferating. Even with increasing concentrations of TGFβ1 accumulating over time in culture and repeated heating of the medium to activate all secreted latent TGFβ1, MATLyLu-pSVTGFβ1 cell proliferation was not reinhibited.

Thus, whether cells were grown in unheated or heat-activated conditioned medium, TGFβ1-overproducing cells became growth inhibited relative to nontransfected or control transfected (pSG5) cells. This suggests that inhibition was indeed due to TGFβ1, rather than to inactivation of growth stimulatory secreted factors. Cells grown in heat-activated medium became inhibited sooner, probably because the concentration of activated TGFβ1 on day 1 was higher in heated medium than in unheated medium. Over time, the concentration of active TGFβ1 in unheated medium became high enough to inhibit growth.

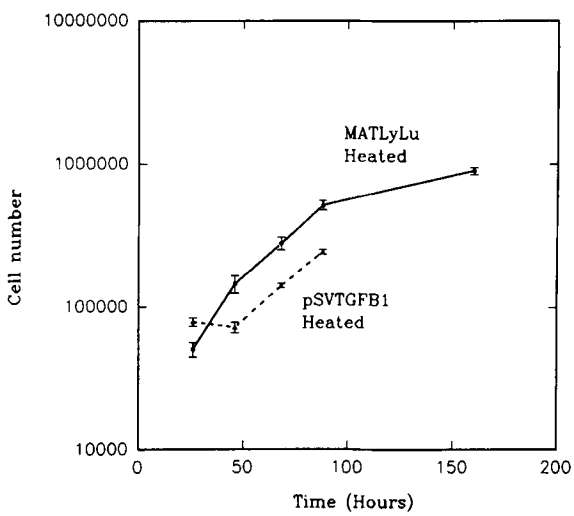

Fig. 7. Growth of TGFβ1-Overproducing MATLyLu-pSVTGFβ1 Cells in Heat-Activated Medium

TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells (*dashed line*) were grown in heat-activated medium, as described in Fig. 5. Nontransfected MATLyLu cells (*solid line*) are shown for comparison. Note the log scale on the y-axis.

TGFβ Antibody Neutralizes the Effect of TGFβ1 Overexpression

In the experiments described above, we attributed the transient *in vitro* growth inhibition of TGFβ1-overproducing transfected MATLyLu cells to TGFβ1 expression, secretion, and activation. To confirm that the effect was indeed due to TGFβ1, we investigated the effect of TGFβ-neutralizing antibody on growth (Table 2). This antibody specifically inhibits the binding of TGFβ to its receptors (R & D Systems, Inc., Minneapolis, MN) (14, 15). TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells were initially growth inhibited and subsequently resumed proliferating, with a 24-h doubling time (Table 2). In contrast, when TGFβ1-overproducing cells were plated in the presence of TGFβ-neutralizing antibody, proliferation was not inhibited, and cells grew. These data confirm that growth inhibition was mediated by TGFβ secreted into the medium. In addition, because the antibody binds only to active TGFβ and not to latent TGFβ (14, 15), the ability of the TGFβ antibody to prevent growth inhibition of MATLyLu-pSVTGFβ1 cells in unheated medium (Table 2) supports our conclusion that the cells are able to activate the TGFβ that they secrete.

DISCUSSION

Prostate cancers express supranormal levels of TGFβ1 mRNA (3), as do many other tumor types (4–9), but until now the relevance of this has been unclear and untested. Using transfected MATLyLu prostate cancer cells, we had the unique opportunity to investigate the

Table 2. Effect of TGFβ1-Neutralizing Antibody on Growth of TGFβ1-Overproducing MATLyLu Cells

Addition	Interval (h)	
	6–18	18–66
None	No growth	24
Ab	11	26

TGFβ1-overproducing MATLyLu-pSVTGFβ1 cells were grown in unheated serum-free medium in 24-well cell culture plates (1 ml/well). TGFβ-neutralizing antibody (Ab) was added at the time of subculture (10 μg/ml) and at each subsequent time point (an additional 5 μg/ml) to neutralize any newly produced activated TGFβ1. Values shown are doubling times (hours), calculated from semilog plots of cell number *vs.* time, as shown in Fig. 6A.

effects of TGFβ1 overexpression *in vivo*. TGFβ1 overexpression in prostate cancer cells *in vivo* had dramatic tumor growth-promoting effects. Compared to controls, TGFβ1-overproducing tumors were significantly larger and less necrotic, and metastatic disease was more extensive. Therefore, TGFβ1 produced *in vivo* is biologically active and can promote prostate cancer growth, viability, and aggressiveness, perhaps via effects on the host and/or on the tumor cells themselves.

The mechanism by which TGFβ1 overexpression enhances MATLyLu tumor size is not known; however, TGFβ1 has several properties, reported in other systems, that may account at least in part for this effect. For example, TGFβ1 is a potent stimulator of angiogenesis (new blood vessel growth) *in vivo* (10, 25). Because tumor size is limited by its blood supply (26), MATLyLu tumors expressing higher levels of TGFβ1 may have higher angiogenesis activity, which might allow tumors to grow to a larger size. Such tumors might also be expected to exhibit less necrosis. TGFβ1 also is a potent suppressor of the immune system (11, 12), and TGFβ1 overexpression in a highly immunogenic murine fibrosarcoma allows it to escape immune surveillance (12).

TGFβ1 has a plethora of effects on the extracellular matrix (1, 2). TGFβ1 can promote its formation and inhibit its degradation (1, 2). It is not clear how these effects, if they occurred in MATLyLu tumors, would confer a growth advantage on TGFβ1-overexpressing tumors, unless an increase in extracellular matrix components provides additional substratum on which cells can continue to proliferate. On the other hand, TGFβ1 also has effects on extracellular matrix that could decrease cell adhesion to or enhance degradation of extracellular matrix components (1, 2); such effects could enhance tumor aggressiveness by facilitating the ability of tumors to metastasize. Thus, TGFβ1 1) stimulates collagenase and heparanase activities in some cell types (13, 27); 2) stimulates production of tenascin (28), a protein component of the extracellular matrix that antagonizes the adhesion of cells to fibronectin (29); and 3) modulates the expression of integrins, cell adhesion molecules that are receptors for extracellular matrix components (30). In MG-63 human osteosarcoma cells, TGFβ1 changed the adhesive phenotype by modifying the expression of integrins (30); this change caused the selective loss of cell adhesion to the basement membrane protein laminin (30). It is interesting in this regard to note that TGFβ1 decreases the adherence of metastatic R3327-AT3 rat prostate cancer cells to tissue culture plastic under certain conditions (19). Thus, decreased cell adhesion to or enhanced degradation of extracellular matrix components might facilitate the ability of TGFβ1-overproducing MATLyLu tumors to metastasize. The angiogenic activity of TGFβ1 might then allow metastatic cells to grow after reaching their destination. This might be reflected biologically by more extensive metastatic disease.

Although TGFβ1 is widely referred to as an inhibitor of epithelial cell proliferation (1, 2, 31), there are exceptions to this generalization. For example, TGFβ1 stimulates *in vitro* proliferation of normal and virus-transformed human neonatal prostate epithelial cells (21) and some cell lines of colon carcinoma (32), melanoma (33), and tracheal cancer (34). TGFβ1 stimulates the production of basic fibroblast growth factor (35, 36), platelet-derived growth factor (PDGF) (37–39), insulin-like growth factor-I (40), and PDGF receptors (41, 42). The mitogenic effect of TGFβ1 on fibroblasts and smooth muscle cells has been attributed to the stimulated expression of PDGF A-chain (37, 38) or PDGF receptor α-subunit (42). Thus, *in vivo*, TGFβ1 produced by epithelial tumor cells could potentially up-regulate growth factor production in the tumor cells (autocrine mechanism) and/or in adjacent nonmalignant supporting stromal cells. Growth factors produced by stromal cells in response to TGFβ1 might, in turn, stimulate tumor cell proliferation (paracrine mechanism).

It is not known whether the tumor growth-enhancing effect of TGFβ1 overexpression *in vivo* is due to the effects of TGFβ1 on the host and/or on the tumor cells. However, because TGFβ1-overproducing MATLyLu cells can activate and respond to secreted latent TGFβ1 *in vitro*, it is reasonable to suspect that effects of TGFβ1 on the tumor cells may account at least in part for effects on the tumor as a whole. Yet, the response *in vitro* was growth inhibition, albeit transient inhibition. Interestingly, proliferation of human prostate cancer cell lines PC3 and DU145 *in vitro* is also inhibited by exogenously added TGFβ1, and over time, these cells resume proliferating at control rates despite retreatment with TGFβ1 (18). Thus, despite continued exposure to TGFβ1, prostate cancer cells lose sensitivity to its inhibitory effect; perhaps this occurs via down-regulation of TGFβ receptors, uncoupling of TGFβ receptors from their signal transduction pathway, or stimulation of growth factors that overcome the growth inhibition. Although the mechanism by which cells escape inhibition is unknown, the transient nature of the inhibition, in both rat and human prostate cancer cells, suggests that TGFβ1 does not have a net growth inhibitory effect on prostate cancer cell proliferation and, as such, is consistent with our *in vivo* data.

The apparent discrepancy between the effects of TGFβ1 *in vivo* and *in vitro* is not surprising in light of other reports that also document growth inhibitory effects of TGFβ1 on cells cultured on plastic *vs.* stimulatory effects *in vivo* (10, 43–45). For example, TGFβ1 inhibits keratinocyte and endothelial cell proliferation *in vitro*, but *in vivo*, it stimulates epidermal growth (43, 45) and angiogenesis (10, 43, 44). TGFβ1 also inhibits hepatocyte proliferation *in vitro*, but *in vivo*, the inhibitory effect is transient, and repeated administration of TGFβ1 at frequent intervals fails to suppress liver regeneration (46). In addition, there are numerous other examples of cells that do not behave *in vitro* as they do *in vivo*, perhaps reflecting the absence *in vitro* of stromal-epithelial interactions, epithelial cell polarity, or other factors (47–49).

In contrast, when endothelial cells are cultured in

three-dimensional collagen gels instead of on plastic, TGFβ1 does not inhibit proliferation; rather, it stimulates cell migration and behavior similar to the angiogenic response *in vivo* (44). In skin explant cultures (where basement membrane-epidermal interactions remain undisrupted), TGFβ1 stimulates growth by stimulating keratinocyte migration from the explant; DNA synthesis is unaffected (45). TGFβ1 inhibits the growth of normal mammary epithelial cells cultured on plastic (39, 50), but not of cells cultured in a collagen gel matrix (50). Therefore, keratinocytes, endothelial cells, and mammary epithelial cells, which prototypically illustrate the growth inhibitory effect of TGFβ1 on epithelial cells, are growth inhibited when cultured on plastic, but not when cultured with extracellular matrix components (44, 45, 50). Similarly, Sertoli cells (48) and uterine epithelial cells (49) cultured on basement membrane Matrigel respond to hormones as they do *in vivo*, whereas these cells on plastic do not respond. Thus, it is not surprising that we have observed different effects of TGFβ1 overexpression *in vivo* and on plastic *in vitro*. Importantly, all of these examples illustrate that growth inhibitory effects of TGFβ1 on cells cultured on plastic may not be predictive of similar effects *in vivo*. However, by studying the *in vivo* behavior of transfected TGFβ1-overproducing prostate cancer cells, we have been able to demonstrate that TGFβ1 is not acting as a prototypical epithelial cell inhibitor in prostate cancer. In addition, by studying *in vitro* behavior, we may infer that the tumor growth-enhancing effect of TGFβ1 *in vivo* may occur at least in part via effects on the tumor cells themselves.

MATERIALS AND METHODS

Cell Culture

The MATLyLu prostate adenocarcinoma cell line was used between passages 128–140; it was originally derived from the Dunning R3327-MATLyLu tumor subline, which is serially transplantable *in vivo*. The properties of the MATLyLu tumor *in vivo* and *in vitro* have been well characterized (16, 17). *In vivo*, MATLyLu cells form a tumor that is poorly differentiated, androgen independent, rapidly growing (1.7-day doubling time), and highly metastatic to lymph nodes and lungs. Cells passaged *in vitro* can be reinoculated *in vivo* to produce a tumor with properties like those of the original *in vivo* tumor (16, 17). For tissue culture, cells were maintained and passaged in serum-containing medium (RPMI-1640 medium containing 10% fetal bovine serum plus 250 nM dexamethasone) and kept at 37 C in a humidified atmosphere of 5% carbon dioxide and 95% air (17). Cell lines were negative for mycoplasma. For experiments in which cells were grown in serum-free medium, cell monolayers were washed in Hanks' Balanced Salt Solution, trypsinized (5 min; 37 C), and subcultured into serum-free medium [Ham's F-12 medium with 1X Nutridoma HU (Boehringer Mannheim, Indianapolis, IN) plus 250 nM dexamethasone]. Cell number was quantitated using a Coulter counter (Hialeah, FL). RPMI-1640 medium, Ham's F-12 medium, fetal bovine serum, and Hanks' Balanced Salt Solution without calcium and magnesium were purchased from Gibco (Grand Island, NY). TGFβ neutralizing antibody was obtained from R & D Systems.

Plasmids

The expression vector pSVTGFβ1 contains a full-length murine TGFβ1 cDNA down-stream of a simian virus-40 (SV40) early promoter (to promote efficient transcription) and up-stream of portions of rabbit β-globin exons 2 and 3 (which do not get translated) and rabbit β-globin polyadenylation signal (12). This plasmid was kindly provided by Dr. R. Daniel Beauchamp, University of Texas Medical Branch. Cells transfected with this vector express high levels of latent TGFβ1 protein (12). The TGFβ1 cDNA fragment (1.6 kb) could be excised from the vector with *Eco*R1 and used to probe cellular RNA or DNA blots for the presence of TGFβ1 mRNA transcripts or TGFβ1 cDNA, respectively. A control vector, pSG5, which contains the SV40 promoter, rabbit globin gene, and rabbit polyadenylation signal was purchased from Stratagene (La Jolla, CA). A third plasmid [pZipneo SV (x)] (51) encoded the gene for resistance to the antibiotic G418 (Geneticin, an aminoglycoside).

Transfection and Selection of TGFβ1 Subclones

Cells transfected with pZipneo become resistant to cell killing by G418. Stable integration of pZipneo into the host genome allows continued proliferation of cells in medium containing G418 (selection medium). MATLyLu cells (passage 128) were cotransfected with the pZipneo plasmid and either the pSVTGFβ1 plasmid or the pSG5 plasmid, using Lipofectin reagent and the protocol described by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). These treated cultures were maintained for 7 weeks in serum-containing medium with 500 μg/ml G418 (Geneticin, Gibco, Grand Island, NY). This concentration is in excess of what is needed to kill all cells that do not contain the pZipneo gene. After this 7-week selection interval, G418-resistant colonies represent clones of cells that have stably integrated the transfected neomycin resistance gene into their genome. Not all colonies that contain pZipneo will also contain the plasmid pSVTGFβ1; thus, further screening is necessary. To select colonies that also have integrated the transfected TGFβ1 murine cDNA, randomly chosen G418-resistant colonies were screened for the presence of pSVTGFβ1 plasmid DNA [a 527-basepair (bp) *Eco*R1/*Xho*I fragment] by Southern DNA analysis and for overexpression of mRNA transcripts by Northern RNA analysis. We identified and isolated a subclone, designated MATLyLu-pSVTGFβ1, which produced high levels of TGFβ1 mRNA. G418-resistant cells that had stably integrated the pSG5 control vector (lacking the TGFβ1 cDNA insert) were detected by probing cellular DNA with a 1.2-kb *Sal*I fragment of the pSG5 plasmid by Southern DNA analysis; this subclone is designated MATLyLu-pSG5.

Southern DNA Analysis

DNA was isolated from 1×10^8 cells, as described previously (52). Purified DNA samples were digested with specific restriction endonucleases at 37 C for 3 h. DNA from MATLyLu-pSG5 was digested with *Sal*I (to probe for the presence of the 1.2-kb *Sal*I fragment of the pSG5 plasmid), and DNA from MATLyLu-pSVTGFβ1 was digested with *Eco*R1 and *Xho*I (to probe for the presence of the 1.6-kb *Eco*R1 TGFβ1 cDNA and the 527-bp *Eco*R1/*Xho*I fragment of pSVTGFβ1 plasmid). DNA was electrophoresed in 0.8% agarose gels, denatured with sodium hydroxide (52), and transferred onto Nytran (Schleicher and Schuell, Inc., Keene, NH) overnight at room temperature in 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7), using the manufacturer's standard protocol. The DNA was baked onto the Nytran at 80 C for 1 h. The membrane was prehybridized for 2 h at 42 C with 50 μg/ml salmon sperm DNA in 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA) plus 10 × Denhardt's solution (1 × Denhardt contains ficoll 400, polyvinylpyrrolidone, and bovine serum

albumin, each at 0.02%) (Sigma, St. Louis, MO) plus 1% SDS. Hybridization with a specific ^{32}P-labeled cDNA probe was carried out for 24 h at 68 C in 6 × SSPE, 1% sodium dodecyl sulfate (SDS), 50% formamide (pH 7.2), 50 μg/ml salmon sperm DNA, and 10% dextran sulfate. The cDNA probes (the 1.2-kb *Sal*I fragment of the pSG5 plasmid, and the 1.6-kb *Eco*R1 fragment and 527-bp *Eco*R1/*Xho*I fragment of the pSVTGFβ1 plasmid) were labeled with deoxycytidine 5′[α-^{32}P] triphosphate (3000 Ci/mmol; Amersham, Arlington Heights, IL) using the Multiprime DNA labeling kit (Amersham International, Amersham, Aylesbury, Buckinghamshire, United Kingdom) based on the method described by Feinberg and Vogelstein (53). After hybridization, the blots were washed as follows: twice in 6 × SSPE-0.1% SDS at room temperature, twice in 1 × SSPE-0.1% SDS at 37 C, and once in 0.1 × SSPE-0.1% SDS at 65 C. The nylon blot was heat-sealed into a plastic bag and placed onto Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70 C for 24–48 h.

RNA Extraction

Total RNA was extracted from either 1 × 10^8 cells or 1 g tumor tissue using a modification of the lithium chloride-urea method (54). Tumor tissue was pulverized in liquid nitrogen and homogenized in 3 M LiCl plus 6 M urea (10 ml/g tissue) at 4 C (30 sec; twice) using a Polytron (Brinkmann, Luzern, Switzerland). Cultured cells were washed with 10 vol Tris-buffered saline (1 M NaCl and 20 mM Tris, pH 7.5) and homogenized in 2 ml 3 M LiCl plus 6 M urea. Homogenates were incubated at 4 C overnight, then centrifuged (SW27 rotor; 25,000 rpm; 30 min). Pellets were resuspended (2 ml/g tissue) in buffer III [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1% SDS], and 3 × FEB [150 mM Tris-HCl (pH 9.0), 3 mM EDTA, and 1.5% SDS] was added (1 ml/g tissue). After gentle mixing, FEB-saturated hot phenol (56 C) was added (2 ml/g tissue) and mixed continuously for 5–10 min. After centrifugation (5,000 rpm; Sorvall HS4 rotor, Norwalk, CT; 30 min; 4 C), the aqueous layer was transferred to a sterile tube, chloroform-isoamyl alcohol (49:1) was added (3 ml/g tissue), and the material was mixed continuously for 5 min and recentrifuged (5,000 rpm; 15 min; 4 C). The aqueous layer was removed, and 0.1 vol 3 M sodium acetate, pH 5.2, plus 2 vol absolute ethanol were added. The RNA was precipitated by cooling for 3 h at −70 C or overnight at −20 C, followed by centrifugation (5,000 rpm; 30 min; 4 C). The pellet was dried under a vacuum and redissolved in 1 ml elution buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA).

Messenger RNA Purification

Poly(A)$^+$ RNA was purified from total RNA using a modification of methods described previously (52) and the spin column method described by Pharmacia (Piscataway, NJ). Total RNA (1 mg in 1 ml elution buffer) was heated at 65 C, placed on ice, mixed gently with 0.2 ml sample buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 3.0 M NaCl], applied to an oligo(dT)-cellulose column (Boehringer Mannheim, Indianapolis, IN), and allowed to seep into the column under gravity. High salt buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5 M NaCl] was applied to the column, and the column was centrifuged at 1375 rpm for 2 min at room temperature in a tabletop centrifuge. The high salt buffer step was repeated, and high salt eluates were discarded. The column was washed three times with low salt buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 M NaCl), centrifuging the column, as described above, after each successive wash; wash eluates were discarded. Poly(A)$^+$ RNA was eluted off the column by applying four successive aliquots of prewarmed elution buffer (65 C) and centrifuging the column (1375 rpm; 2 min) after each application. The combined eluate was reheated, 0.2 ml sample buffer was added, and the mixture was reapplied to the oligo(dT)-cellulose column; all subsequent steps were repeated as described above. The final poly(A)$^+$ RNA eluate was transferred to a sterile tube and precipitated by the addition of 0.1 vol sample buffer, 0.01 vol glycogen (10 mg/ml) as a coprecipitant, and 2.5 vol cold absolute ethanol. After cooling at −20 C overnight, the sample was centrifuged at 5000 rpm for 30 min at 4 C. The poly(A)$^+$ RNA precipitate was dissolved in elution buffer and stored at −70 C. The concentration of RNA was determined spectrophotometrically.

Northern RNA Analysis

Poly(A)$^+$ RNA was electrophoresed in a 1.7% agarose gel containing 2.2 M formaldehyde (55), then transferred overnight onto nylon (Nytran, Schleicher and Schuell) in the presence of 10 × SSC buffer. Blots were washed (5 × SSC buffer; 5 min), air dried, baked (80 C; 1 h), prehybridized (68 C; 1 h; in 5 × SSC, 5× Denhardt's solution, 100 μg/ml yeast transfer RNA, and 0.1% SDS), and then hybridized at 68 C overnight in prehybridization solution containing 1 × 10^6 cpm/ml ^{32}P-labeled cDNA. Blots were washed twice with 1 × SSC and 0.1% SDS (22 C; 15 min), and twice with 0.25 × SSC and 0.1% SDS (68 C; 30 min), then placed onto Kodak XAR-5 film at −70 C for 1–7 days. Murine TGFβ1 cDNA (1.6 kb) was excised from the pSVTGFβ1 plasmid using *Eco*RI, purified by electrophoresis in agarose, and labeled with [^{32}P]dCTP using the Multiprime DNA labeling kit (Amersham).

Tumor Transplantation

Before inoculating cultured cells into animals, cells grown in serum-containing medium were washed with Hanks' buffer, and trypsinized for 5 min at 37 C. Cells were collected in Hanks' buffer and centrifuged at 1000 rpm for 10 min. The cells were resuspended in Hanks' buffer at a concentration of 1 × 10^6 cells/0.3 ml. Adult male Copenhagen rats (300 g BW; 60 days old; from Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with Metofane (methoxyflurane, Pitman-Moore, Washington Crossing, NJ), and 0.3 ml cell suspension was injected sc into each flank. Tumor size was measured in two dimensions using calipers, and tumor volume was calculated according to the formula: volume = 0.5 (length + width) × length × width × 0.5236 (16).

Quantitation of Metastatic Disease

Metastatic disease was quantitated macroscopically. Lung metastases were surveyed in each of the five anatomical lobes: right upper, right middle, right lower, left upper, and left lower. The presence of metastasis in a single lobe was designated as 20% involvement. Retroperitoneal and axillary lymph nodes were evaluated for the presence or absence of metastasis.

Statistical Analysis

The statistical significance of differences between groups was analyzed using Student's *t* test. Data are presented as the mean ± SEM.

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